



Europäisches
Patentamt

European
Patent Office

Office européen
des brevets

REC'D 12 DEC 1997

WIPO

PCT

Bescheinigung

Certificate

Attestation

Die angehefteten Unterla-
gen stimmen mit der
ursprünglich eingereichten
Fassung der auf dem näch-
sten Blatt bezeichneten
europäischen Patentanmel-
dung überein.

The attached documents
are exact copies of the
European patent application
described on the following
page, as originally filed.

Les documents fixés à
cette attestation sont
conformes à la version
initialement déposée de
la demande de brevet
européen spécifiée à la
page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

97100584.8

PRIORITY DOCUMENT

Der Präsident des Europäischen Patentamts:
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

Ch. Dufrasne

DEN HAAG, DEN
THE HAGUE,
LA HAYE, LE

01/12/97



Europäisches
Patentamt

European
Patent Office

Office européen
des brevets

Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

Anmeldung Nr.:
Application no.: 97100584.8
Demande n°:

Anmeldetag:
Date of filing: 16/01/97
Date de dépôt:

Anmelder:
Applicant(s):
Demandeur(s):
BOEHRINGER MANNHEIM GMBH
68298 Mannheim
GERMANY

Bezeichnung der Erfindung:
Title of the invention:
Titre de l'invention:
Thermostable nucleic acid polymerase from *Thermococcus gorgonarius*

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

Staat: EP
State:
Pays:

Tag: 02/10/96
Date:
Date:

Aktenzeichen:
File no
Numéro de dépôt:

EPA96115874

Internationale Patentklassifikation:
International Patent classification:
Classification internationale des brevets

C12N15/52, C12N9/12, C12N15/70, C12N1/21, C12P21/00

Am Anmeldetag benannte Vertragsstaaten.
Contracting states designated at date of filing: AT/BE/CH/DE/DK/ES/FI/FR/GB/GR/IE/IT/LI/LU/MC/NL/PT/SE
Etats contractants désignés lors du dépôt:

Bemerkungen:
Remarks:
Remarques:

Thermostable nucleic acid polymerase from *Thermococcus gorgonarius*5

The present invention relates to an extremely thermostable enzyme. More specifically, it relates to a thermostable DNA polymerase obtainable from *Thermococcus gorgonarius*.

10

DNA polymerases are a family of enzymes which are in particular involved in DNA replication and repair. Extensive research has been conducted on the isolation of DNA polymerases from mesophilic microorganisms such as *E.coli* (see, for example, Bessman et al. (1957) *J. Biol. Chem.* 223:171-177, and Buttin and Kornberg, (1966) *J. Biol. Chem.* 241:5419-5427).

15

Research has also been conducted on the isolation and purification of DNA polymerases from thermophiles, such as *Thermus aquaticus* (Chien, A., (1976) et al. *J. Bacteriol.* 127:1550-1557) Further, the isolation and purification of a DNA polymerase with a temperature optimum of 80°C from *Thermus aquaticus* YT1 strain has been described (EP 0 258 017 and US

20

4, 889, 819). Research has indicated that while the Taq DNA polymerase has a 5'-3' polymerase-dependent exonuclease function, the Taq DNA polymerase does not possess a 3'-5' proofreading exonuclease function (Lawyer, F.C. et al., (1989) *J. Biol. Chem.*, 264:6427-6437. Bernad A., et al. (1989) *Cell* 59:219). As a result, Taq DNA polymerase is prone to base incorporation errors, making its use in certain applications undesirable. For example, attempting to clone an amplified gene is problematic since any one copy of the gene may contain an error due to a random misincorporation event. Depending on where in the PCR cycle that error occurs (e.g., in an early replication cycle), the entire DNA amplified could contain the erroneously incorporated base, thus, giving rise to a mutated gene product. Furthermore, research has indicated that Taq DNA polymerase has a thermal stability of not more than several minutes at 100°C.

30

The 3'-5' exonuclease activity is generally considered to be desirable, because misincorporated or unmatched bases of the synthesized nucleic acid sequence are eliminated by this activity. Therefore, the fidelity of PCR utilizing a polymerase with 3'-5' exonuclease activity is increased. Such an enzyme is, e.g. the DNA polymerase from *Pyrococcus furiosus* (Lundberg et al., (1991) *Gene.*, 108; p. 1-6).

Other more recent investigation focusses on the isolation and purification of DNA polymerases from archaebacteria such as *Thermococcus sp.* (EP 0 455 430), in particular a purified DNA polymerase obtainable from *Thermococcus litoralis* is described. Also the recombinant preparation and the gene encoding for this enzyme is known in the art (EP 0 547 920).

In EP 0 455 430 is also described a DNA polymerase from *Pyrococcus sp.* and the gene thereof which also contains introns to be removed for expression of the functional enzyme in *E.coli*.

Accordingly, there is a desire in the art to obtain and produce a purified, highly thermostable DNA polymerase with 3'-5' proofreading exonuclease activity suitable to improve the PCR process.

The present invention meets this need by providing a DNA polymerase from *Thermococcus gorgonarius* (Tgo) together with the related DNA and amino acid sequence information, recombinant expression vector and a purification protocol for said DNA polymerase. The DNA polymerase according to the present invention exhibits more than a two fold greater replication fidelity than known DNA polymerases, e.g. obtainable from *Pyrococcus furiosus*. A further advantage is that the 3'-5' exonuclease activity found in *T. gorgonarius* polymerase can also decrease non-specific background amplification in PCR by degrading defrayed ends of primers bound to unspecific sequences thereby destabilizing the binding of the primer because of decreasing the length of the helix. Tgo polymerase is thus unexpectedly superior to known DNA polymerases in amplification protocols requiring high fidelity DNA synthesis. Another advantageous property of the DNA polymerase of *Thermococcus gorgonarius* is the fact, that

the gene does not contain intervening sequences which would have to be removed to accomplish expression in *E.coli*.

The thermostable DNA polymerase enzyme obtainable from *T. gorgonarius* catalyzes the
5 template directed polymerization of DNA, has an apparent molecular weight of about 92,000-
96,000 daltons and retains 90 % of its activity after incubation for two hours at 95°C in the
presence of a stabilizer like a non-ionic detergent as, e. g., 0.01 % Thesit™
(Dodecylpoly(ethylenglycolether)_n) or 0.01 % Nonidet P40™
(Ethylphenolpoly(ethylenglycolether)_n).

10 Moreover, DNA encoding the 92,000-96,000 daltons thermostable DNA polymerase obtain-
able from *Thermococcus gorgonarius* has been isolated and which allows to obtain the ther-
mostable enzyme of the present invention by expression in *E.coli*. The DNA sequence of the
DNA polymerase obtainable from *Thermococcus gorgonarius* is shown in SEQ ID No. 6. The
15 recombinant *Thermococcus gorgonarius* DNA polymerase also possesses 3'-5' exonuclease
(proofreading) activity. Furthermore the gene encoding DNA polymerase from *Thermococcus*
gorgonarius does not contain intervening sequences.

20 *Thermococcus gorgonarius* was isolated from E. A. Bonch-Osmolovskaya and V. A. Svet-
lichny, Institute of Microbiology, Russian Academy of Sciences, Moscow, Russia. *Thermo-*
coccus gorgonarius is a new strain, isolated from a thermal vent in New Zealand. This strain
does not show DNA-DNA homology with *T. celer*, *T. litoralis* or *T. stetteri* (E.A. Bonch-
Osmolovskaya, unpublished results).

25 The preferred thermostable enzyme herein is a DNA polymerase obtainable from *Thermococ-*
cus gorgonarius DSM 8976 (deposited on the Deutsche Sammlung von Mikroorganismen und
Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig). This organism is an
extremely thermophilic, sulfur metabolizing, archaebacterium, with a growth range between
55°C and 98°C.

A preferred method for isolation and purification of the enzyme is accomplished - after all growth - using the multi-step process as follows:

First, the frozen cells are thawed, suspended in a suitable buffer such as buffer A (40 mM Tris-HCl buffer, pH 7.4; 0.1 mM EDTA, 7 mM 2-mercaptoethanol; 1 mM Pefabloc SC™ (4-(2-Aminoethyl)-benzolsulfonylfluorid)), disrupted by high pressure at 1.200 bar. KCl was added to the extract to a final concentration of 400 mM and the solution cleared by centrifugation. The supernatant is then passed through a Heparin Sepharose Cl 6B column (Pharmacia), which has a strong affinity for nucleic acid binding proteins. The nucleic acids present in the supernatant solution of *Thermococcus gorgonarius* and many of the other proteins pass through the column and are removed by washing the column with two column volumes of buffer A. After washing, the enzyme is eluted with a linear gradient from 0 to 1 M NaCl in buffer A. The peak DNA polymerase activity is dialyzed and applied to a DEAE Sephacel column (Pharmacia). The column is washed with buffer A and the enzyme activity eluted with a linear gradient from 0 to 1 M NaCl in buffer A. The peak DNA polymerase activity is dialyzed and applied to a Cellulose Phosphate column (Whatman). The enzyme is again eluted with a linear gradient such as 0 to 1 M NaCl in buffer A. The enzyme is about 40 % pure at this stage.

The apparent molecular weight of the DNA polymerase obtainable from *Thermococcus gorgonarius* is between about 92,000 to 96,000 daltons when compared with DNA polymerases of known molecular weight, such as *E.coli* DNA polymerase I and *Thermus thermophilus* DNA polymerase. It should be understood, however, that as a protein from an extreme thermophile, *Thermococcus gorgonarius* DNA polymerase may migrate during electrophoresis at an aberrant relative molecular weight due to failure to completely denature or other intrinsic properties. The exact molecular weight of the thermostable enzyme of the present invention may be determined from the coding sequence of the *Thermococcus gorgonarius* DNA polymerase gene. The molecular weight of the DNA polymerase may be determined by any technique, for example, by *in situ* analysis after separation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described in Spanos, A. and Hübscher, U., (1983) *Methods in Enzymology* 91:263-277.

Polymerase activity is either measured by the incorporation of radioactively labeled deoxynucleotides into DNase-treated, or activated DNA, following subsequent separation of the unincorporated deoxynucleotides from the DNA substrate. Polymerase activity is proportional to the amount of radioactivity in the acid-insoluble fraction comprising the DNA, as described by Lehman, I.R., et al. (1958) *J. Biol. Chem.* 233:163, or by incorporation of digoxigenin-labeled dUTP and determination of incorporated Digoxigenin-dUTP using chemoluminescence according to the method described in Hölte, H.-J.; Sagner, G; Kessler, C.; and Schmitz, G., (1992) *Biotechniques* 12:104 -113.

The DNA polymerase of the present invention has a very high thermal stability at 95°C. It retains approximately 90 percent of its activity after incubation at 95°C for 120 minutes in the presence of stabilizer. The thermal stability is determined by preincubating the enzyme at the temperature of interest in the presence of all assay components (buffer, MgCl₂, deoxynucleotides, activated DNA and a stabilizer like 0.01 % Thesit™ and 0.01 % Nonidet P40™) except the single radioactively-labeled deoxynucleotide. At predetermined time intervals, ranging from 1-120 minutes, small aliquots are removed, and assayed for polymerase activity using one of the methods described above.

The thermostable enzyme of this invention may also be produced by recombinant DNA techniques, as the gene encoding this enzyme has been cloned from *Thermococcus gorgonarius* genomic DNA. The complete coding sequence for the *Thermococcus gorgonarius* DNA polymerase can be derived from the plasmid pBTac2Tgo on an approximately 2.3 kB EcoRI/PstI restriction fragment.

The production of a recombinant form of *Thermococcus gorgonarius* DNA polymerase generally includes the following steps: DNA is isolated which codes for the active form of the polymerase. This can be accomplished e.g. by screening of a DNA library derived from the genomic DNA of *T. gorgonarius* using the DNA sequence described in SEQ ID No.: 1 as a probe. Clones containing DNA fragments of *T. gorgonarius* hybridizing to the probe are isolated and the nucleotide sequence of the plasmid inserts determined. Complete isolation of the coding region and the flanking sequences of the DNA polymerase gene can be performed by

restriction fragmentation of the *T. gorgonarius* DNA with another restriction enzyme as in the first round of screening and by inverse PCR (Innis et al., (1990) *PCR Protocols*; Academic Press, Inc., p. 219-227). This can be accomplished with synthesized oligonucleotide primers binding at the outer DNA sequences of the gene part but in opposite orientation e.g. with the
5 SEQ ID Nos. 2 and 3. As template *T. gorgonarius* DNA is used which is cleaved by restriction digestion and circularized by contacting with T4 DNA ligase. To isolate the coding region of the whole polymerase gene, another PCR is performed using primers as shown in SEQ ID Nos. 4 and 5 to amplify the complete DNA polymerase gene directly from genomic DNA and introducing ends compatible with the linearized expression vector.

10

SEQ ID NO. 1:

5'-ATG ATH YTN GAY ACN GAY TAY ATH AC-3'

SEQ ID NO. 2:

15 5'-GGC CTA CGA GAG GAA CGA ACT GGC-3'

SEQ ID NO. 3:

5'-GGC GTA GAT GTA GGG CTC-3'

20 SEQ ID NO. 4:

5'-GAG CTG GTC GAA TTC ATG ATC CTG GAC GCT GAC TAC ATC ACC -3'

SEQ ID NO. 5:

5'- AGC CTG CAG TCA TGT CTT AGG TTT TAG CCA CGC-3'

25

The gene is operably linked to appropriate control sequences for expression in either prokaryotic or eucaryotic host/vector systems. The vector preferably encodes all functions required for transformation and maintenance in a suitable host, and may encode selectable markers and/or control sequences for polymerase expression. Active recombinant thermostable polymerase can be produced by transformed host cultures either continuously or after
30

be used for lambda gt11 lysogeny. The transformed cells are preferably grown at 37°C and expression of the cloned gene is induced with IPTG (Isopropyl-β-D-thiogalactopyranosid).

Isolation of the recombinant DNA polymerase can be performed by standard techniques. Separation and purification of the DNA polymerase from the *E. coli* extract can be performed by standard methods. These methods include, for example, methods utilizing solubility such as salt precipitation and solvent precipitation, methods utilizing the difference in molecular weight such as dialysis, ultra-filtration, gel-filtration, and SDS-polyacrylamide gel electrophoresis, methods utilizing a difference in electric charge such as ion-exchange column chromatography, methods utilizing specific interaction such as affinity chromatography, methods utilizing a difference in hydrophobicity such as reversed-phase high performance liquid chromatography and methods utilizing a difference in isoelectric point such as isoelectric focussing electrophoresis.

One preferred method for isolating and purification of the recombinant enzyme is accomplished using the multi-stage process as follows.

The frozen cells are thawed and suspended in a suitable buffer such as buffer A (40 mM Tris-HCl, pH 7.5; 0.1 mM EDTA; 7 mM 2-mercaptoethanol) in the presence of Pefabloc SC in a final concentration of 1 mM, lysed by the addition of lysozyme (200 µg/ml) under stirring for 30 min. at 4°C. Sodium deoxycholate is added to a final concentration of 0.05 %. After an incubation for another 30 min. KCl is added to a final concentration of 0.75 M. The suspension is incubated at 72°C for 15 min. and centrifuged. The supernatant is adjusted to 25 % saturation with (NH₄)₂SO₄ and then applied to a hydrophobic interaction chromatography column such as TSK Butyl Toyopearl 650C (TosoHaas). Most of the nucleic acids and unspecific proteins are in the flow through and wash of the column while the polymerase is eluting at the end of a decreasing gradient from 30 % to 0 % saturation of (NH₄)₂SO₄ in buffer A (with additional 10 % glycerol). The polymerase-active fractions are pooled, dialyzed against buffer A containing 10 % glycerol, adjusted to 10 mM MgCl₂ and applied to a high affinity column for nucleotide-binding enzymes such as Fractogel TSK AF-Blue column (Merck). The column is washed with buffer A containing 10 % glycerol and the polymerase protein is eluted with a linear gradient of 0 to 3 M NaCl in buffer A (with additional 10 % glycerol). The

induction of expression. Active thermostable polymerase can be recovered either from host cells or from the culture media if the protein is secreted through the cell membrane.

It is also preferable that *Thermococcus gorgonarius* thermostable polymerase expression is tightly controlled in *E.coli* during cloning and expression. Vectors useful in practising the present invention should provide varying degrees of controlled expression of *Thermococcus gorgonarius* polymerase by providing some or all of the following control features: (1) promoters or sites of initiation of transcription, either directly adjacent to the start of the polymerase gene or as fusion proteins, (2) operators which could be used to turn gene expression on or off, (3) ribosome binding sites for improved translation, and (4) transcription or translation termination sites for improved stability. Appropriate vectors used in cloning and expression of *Thermococcus gorgonarius* polymerase include, for example, phage and plasmids. Example of phage include lambda gt11 (Promega), lambda Dash (Stratagene) lambda ZapII (Stratagene). Examples of plasmids include pBR322, pBTac2 (Boehringer Mannheim), pBluescript (Stratagene), pSP73 (Promega), pET3A (Rosenberg, A.H. et al., (1987) *Gene* 56:125-135) and pET11C (Studier, F. W. et al. (1990) *Methods in Enzymology*, 185:60-89). According to the present invention the use of a plasmid has shown to be advantageously, particularly pBTac2. The Plasmid pBTac2 carrying the *Thermococcus gorgonarius* DNA polymerase gene is then designated pBTac2Tgo.

Standard protocols exist for transformation, phage infection and cell culture (Maniatis, et al. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbour Laboratory Press). Of the numerous *E.coli* strains which can be used for plasmid transformation, the preferred strains include JM110 (ATCC 47013), LE392 pUBS 520 (Maniatis et al. *supra*; Brinkmann et al., (1989) *Gene* 85:109-114;), JM101 (ATCC No. 33876), XL1 (Stratagene), and RR1 (ATCC no. 31343), and BL21 (DE3) plysS (Studier, F. W. et al., (1990) *Methods in Enzymology, supra*). According to the present invention the use of the *E. coli* strain LE392 pUBS 520 has shown to be advantageously. The *E. coli* strain LE392 pUBS 520 transformed with the plasmid pBTac2Tgo is then designated *E. coli* pBTac2Tgo (DSM No. 11328). *E.coli* strain XL1 Blue (Stratagene) is among the strains that can be used for lambda phage, and Y1089 can

Lane 4: 10 μ l of fraction obtained after second chromatography step (Fractogel Blue).

Lane 5: 10 units of DNA polymerase from *Thermococcus gorgonarius*.

Lane 6: Molecular weight markers

Lane 7: 10 units of DNA polymerase from *Pyrococcus woesei*.

5 Lane 8: Molecular weight markers

Figure 2:

In situ activity analysis of native and recombinant *Thermococcus gorgonarius* DNA polymerase in comparison to Klenow fragment, Pol I of *E.coli* and *Thermus thermophilus* DNA polymerase as described in Example I. Native and recombinant *Thermococcus gorgonarius* DNA polymerase have the same electrophoretic mobility.

10

Figure. 3:

DNA sequence and the deduced amino acid sequence of the gene encoding the DNA polymerase from *Thermococcus gorgonarius*.

15

Figure. 4:

Determination of heat stability of *T.gorgonarius* polymerase as described in Example V.

Figure. 5:

Analysis of 3'-5' exonuclease activity as described in Example VI.

Various amounts (units are indicated in the figure) of *T.gorgonarius* DNA polymerase were incubated with DNA fragments in the absence (- dNTPs) and presence (+dNTPs) of deoxynucleotide triphosphates. ctrl1 and 2: Control reactions without DNA polymerase.

25

The 3'-5' exonuclease activity is dependent on the presence or absence of dNTPs.

polymerase fractions are pooled and dialyzed against the storage buffer B (20 mM Tris-HCl, pH 8.0; 0.1 mM EDTA; 10 mM 2-mercaptoethanol; 50 mM $(\text{NH}_4)_2\text{SO}_4$; 50 % glycerol) and stored at -20°C .

- 5 The *Thermococcus gorgonarius* DNA polymerase of the present invention may be used for any purpose in which such an enzyme is necessary or desirable. For example, in recombinant DNA technology including, second-strand cDNA synthesis in cDNA cloning and DNA sequencing. See Maniatis, et al., *supra*.
- 10 The *Thermococcus gorgonarius* DNA polymerase of the present invention may be modified chemically or genetically - site directed or random - to inactivate the 3'-5' exonuclease function and used for any purpose in which such a modified enzyme is desirable, e.g., DNA sequencing or DNA labelling.
- 15 In addition, the *Thermococcus gorgonarius* DNA polymerase of the present invention may also be used to amplify DNA, e.g., by the procedure disclosed in EP 0 200 362, EP 0 201 184 and EP 0 693 078.

The following examples are given to illustrate embodiments of the present invention as it is presently preferred to practice. It will be understood that the examples are illustrative, and that
20 the invention is not be considered as restricted except as indicated in the appended claims.

Brief description of the drawings

- 25 Figure 1:
SDS polyacrylamide gel analysis of partially purified and purified recombinant DNA polymerase from *T. gorgonarius*.
Lane 1: 1 μl of crude extrat.
Lane 2: 5 μl of polymerase fraction obtained after the first chromatography step (TSK Butyl
30 Toyopearl 650C)
Lane 3: 5 μl of fraction obtained after second chromatography step (Fractogel Blue).

Example I

Purification of a thermostable DNA polymerase from *Thermococcus gorgonarius*

5 *Thermococcus gorgonarius* (DSM 8976) was grown in the medium which was prepared as follows: A mineral solution containing KCl, 325 mg/l; $\text{MgCl}_2 \cdot 2 \text{H}_2\text{O}$, 2.75 mg/l; $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 3.45 mg/l; NH_4Cl , 0.25 mg/l; $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$, 0.15 mg/l; KH_2PO_4 , 0.15 mg/l; NaCl, 18 g/l; NaHCO_3 , 1 g/l; trace elements, 4 ml/l (Balch et al., (1979) *Microbiol. Rev.* 43:260), vitamins, 4 ml/l (Balch supra.); Rezazurin, 1 mg/l; 0.4 ml/l of a 0.2 % solution of

10 $\text{Fe}(\text{NH}_2)_2(\text{SO}_4)_2 \cdot 7 \text{H}_2\text{O}$ was boiled and cooled. The following components were added to the final concentrations as indicated: Peptone, 5 g/l; yeast extract, 1 g/l; $\text{Na}_2\text{S} \cdot 9 \text{H}_2\text{O}$, 250 mg/l and cystein-HCl, 250 mg/l, the pH was adjusted to 6.2 - 6.4. The incubation temperature was 88°C. The cells were cooled to room temperature, collected by centrifugation and stored at -70°C. 6 g of cells were suspended in 12 ml of buffer A (40 mM Tris-HCl, pH 7.5; 0.1 mM

15 EDTA; 7 mM 2-mercaptoethanol) containing 1 mM Pefabloc SC™ and disrupted by pressure at 1200 bar. KCl was added to a final concentration of 400 mM, dissolved and the solution was centrifugated at 48,200 x g for 30 minutes at 4°C. The supernatant was passed through a 31 ml Heparin Sepharose Cl 6B column (Pharmacia). The column was then washed with 62 ml of buffer B (buffer A containing 10 % glycerol). The column was eluted with a 310 ml

20 linear gradient from 0 to 1.0 M NaCl in buffer B. The DNA polymerase eluted between 30 and 45 mS/cm. The fractions containing DNA polymerase activity were pooled and dialyzed twice against 600 ml buffer B respectively and applied to a 18 ml DEAE Sephacel column (Pharmacia). The column was washed with two column volumes of buffer B, and eluted with a 160 ml linear gradient of 0 to 0.9 M NaCl in buffer B. The polymerase activity eluted

25 between 4 and 14 mS/cm. Fractions were pooled, dialyzed twice against buffer B (200 ml each time) and applied to a 4 ml Cellulose Phosphate P11 column (Whatman). The column was washed with 8 ml of buffer B and the activity eluted with a 40 ml linear gradient of 0 to 1 M NaCl. The active fractions which eluted between 13 and 32 mS/cm were pooled, dialyzed against buffer B containing $(\text{NH}_4)_2\text{SO}_4$ to 25% saturation and applied to a 4 ml TSK Butyl

30 Toyopearl 650C column (TosoHaas). The column was washed with 8 ml 25% $(\text{NH}_4)_2\text{SO}_4$ -saturated buffer B and eluted with 40 ml of a decreasing gradient of 25 % to 0 % $(\text{NH}_4)_2\text{SO}_4$ -

saturated buffer B. The polymerase eluted between 74 and 31 mS/cm, the pool was dialyzed against buffer B and applied to a 4 ml Fractogel TSK AF-Orange column (Merck). The column was washed with 8 ml of buffer B and eluted with a 80 ml linear gradient of 0 to 2.0 M NaCl. The active fractions (between 76 and 104 mS/cm) were pooled and dialyzed
5 against storage buffer C (20 mM Tris-HCl, pH 8.0; 0.1 mM EDTA; 10 mM 2-mercaptoethanol; 50 mM $(\text{NH}_4)_2\text{SO}_4$; 50 % glycerol) and stored at -20°C . At this step the DNA polymerase was approximately 40 % pure.

The molecular weight of the isolated DNA polymerase was determined by "activity gel analysis" according to a modified version of the method described by Spanos, A. and Hübscher, U.,
10 *supra*. The DNA polymerase sample was separated on a SDS polyacrylamide gel containing activated calf thymus DNA. The polymerase was renatured in the gel in 50 mM Tris-HCl, pH 8.8; 1 mM EDTA; 3 mM 2-mercaptoethanol; 50 mM KCl; 5 % glycerol. Labeling of the DNA with Dig-dUTP (Boehringer Mannheim) was performed in 10 ml of the following
15 buffer: 50 mM Tris-HCl, pH 8.8; 7 mM MgCl_2 ; 3 mM 2-mercaptoethanol; 100 mM KCl; 12 μM dGTP; 12 μM dCTP; 12 μM dATP; 6 μM dTTP; 6 μM Dig-dUTP. The gel was first incubated under shaking at room temperature (30 min.) and then slowly warmed up to 72°C by temperature increments of 5°C . At each temperature interval DNA synthesis is allowed to proceed for 30 min., in order to detect also polymerase activity of mesophile control poly-
20 merases. Then the gel was washed and the DNA was blotted on a nylon membrane (Boehringer Mannheim), UV crosslinked. The digoxigenin labeled DNA was detected using the protocol described in the "Boehringer Mannheim's Dig System User's Guide for Filter Hybridization". As molecular weight markers *E.coli* DNA polymerase I, *Thermus thermophilus* DNA polymerase and Klenow fragment were analyzed on the same gel. The DNA
25 polymerase isolated from *Thermococcus gorgonarius* has an apparent molecular weight in the range of 92,000 to 96,000 daltons as shown in figure 2.

Example 2

Cloning of the *T. gorgonarius* DNA polymerase

- 5 1. DNA from *T. gorgonarius* was isolated and purified by the method described in Lawyer, F. C. et al. (1989) *J. Biol. Chem.* 264:6427-6437.
2. The DNA was restricted with BamHI, separated on an low melting point agarose gel, denatured and blotted onto a nylon membrane. The blot was probed with a Digoxigenin
10 labeled oligonucleotide of the sequence shown in SEQ ID No. 1. A signal could be detected and the region corresponding to the hybridization signal was cut out of the gel. The gel piece was melted and the DNA isolated by ethanol precipitation.
3. The DNA fragments isolated were ligated into a plasmid vector, hybridized with SEQ ID.
15 No. 1. The plasmid DNA from positive clones were isolated and the nucleic acid sequences of the insert determined. The DNA sequences obtained were then compared with sequences of DNA polymerase genes published in Braithwaite, D. K. and Ito J.(1993), *Nucl. Acids Res.* 21:787-802.
- 20 4. From the sequence of one of the cloned fragments which showed a high degree of homology to the B type DNA polymerases described in the publication of Braithwaite et al., *supra*, the primers SEQ ID No. 2 and 3 were designed. These primers bind close to the ends of the cloned DNA fragment in opposit orientations to allow amplification of the flanking genomic sequences in circularized template DNA.
- 25 5. With these primers "inverse PCR" was performed according of the method of Innis, M. A., *supra*, with the DNA from step 1 which was cleaved with EcoRI and circularized with T4 DNA ligase. With this technique two fragments were generated and the sequences determined. An open reading frame could be identified. The deduced aminoacid sequence
30 showed strong homologies to known DNA polymerases of the pol B type.

6. From the sequence of the DNA fragment identified in step 5 new primers were designed, the sequences are shown in SEQ ID No. 4 and 5 which were complementary to the start and the end of the reading frame. The primers contained additional non complementary 5' sequences with restriction sites to introduce clonable ends into the PCR product in such an orientation that the product would be under transcriptional and translational control of the promoter.

7. The PCR product was cleaved with EcoRI and PstI, purified and ligated into the vector pBTac2. This clone, expressing the DNA polymerase from *Thermococcus gorgonarius* was designated pBTac2Tgo.

SEQ ID NO. 1:

5'-ATG ATH YTN GAY ACN GAY TAY ATH AC-3'

SEQ ID NO. 2:

5'-GGC CTA CGA GAG GAA CGA ACT GGC-3'

SEQ ID NO. 3:

5'-GGC GTA GAT GTA GGG CTC-3'

SEQ ID NO. 4:

5'-GAG CTG GTC GAA TTC ATG ATC CTG GAC GCT GAC TAC ATC ACC -3'

SEQ ID NO. 5:

5'-AGC CTG CAG TCA TGT CTT AGG TTT TAG CCA CGC-3'

Example 3

Expression of recombinant *T. gorgonarius* DNA

- 5 The vector from example 2 was transformed into *E.coli* strain LE 392 pUBS 520, cultivated in a fermentor in a rich medium containing the appropriate antibiotic. Induction was performed at an optical density of 1.25 A₅₄₀ with 0.5 mM IPTG. The DNA polymerase from *T. gorgonarius* may also be cloned and expressed by other methods.
- 10 Cells are harvested at an optical density of 5.4 A₅₄₀ by centrifugation and frozen until needed or lyzed by treatment with lysozyme to produce a crude cell extract containing the *T. gorgonarius* DNA polymerase activity.

The crude extract containing the *T. gorgonarius* DNA polymerase activity is purified by the method described in Example 1, or by other purification techniques such as affinity-chromatography, ion-exchange-chromatography or hydrophobic-interaction-chromatography.

15

Example 4

20

Purification of recombinant *T. gorgonarius* DNA Polymerase

E.coli (LE392 pUBS520) pBtac2Tgo (DSM No. 11328) was grown in a 10 l fermentor in media containing 20 g/liter tryptone, 10 g/liter yeast extract, 5 g/liter NaCl and 100 mg/liter ampicillin at 37°C and induced with 0.5 mM IPTG at midexponential growth phase and incubated an additional 4 hours. About 45 g of cells were harvested by centrifugation and stored at -70°C.

25

2 g of cells were thawed and suspended at room temperature in 4 ml of Buffer A (40 mM Tris-HCl, pH 7.5; 0.1 mM EDTA; 7 mM 2-mercaptoethanol; 1 mM Pefabloc SC). 1.2 mg of lysozyme were added and the cells were lyzed under stirring for 30 minutes at 4°C. 4.56 mg

30

sodium deoxycholate were added and the suspension incubated for 10 minutes at room temperature followed by 20 minutes at 0°C. The crude extract was adjusted to 750 mM KCl, heated for 15 minutes at 72°C and centrifuged for removal of denatured protein.

- 5 The supernatant was adjusted to 25 % saturation with $(\text{NH}_4)_2\text{SO}_4$ and applied to a TSK Butyl Toyopearl 650C column (1.5 x 10 cm; 17.7 ml bed volume) equilibrated with buffer B (buffer A containing 10 % glycerol) and 30 % $(\text{NH}_4)_2\text{SO}_4$ -saturation. The column was washed with 70 ml of buffer B and the polymerase was eluted with a 177 ml linear gradient of buffer B containing 30 % to 0 % $(\text{NH}_4)_2\text{SO}_4$ saturation and 0 to 0.2 % Thesit™ (v/v).

10

- The column fractions were assayed for DNA polymerase activity. DNA polymerase activity was measured by incorporation of digoxigenin labeled dUTP into the newly synthesized DNA and detection and quantification of the incorporated digoxigenin essentially as described below. The reaction is performed in a reaction volume of 50 µl containing 50 mM Tris-HCl, pH 8.5; 15 mM $(\text{NH}_4)_2\text{SO}_4$; 7 mM MgCl_2 ; 10 mM 2-mercaptoethanol; 100 µM of dATP, dGTP, dCTP, dTTP, respectively; 200 µg/ml BSA; 12 µg of DNase activated DNA from calf thymus and 0.036 µM digoxigenin-dUTP and 1 or 2 µl of diluted (0.05 U to 0.01 U) DNA polymerase from *T. gorgonarius*. The samples are incubated for 30 min. at 72°C, the reaction is stopped by addition of 2 µl of 0.5 M EDTA, and the tubes placed on ice. After addition of 8 µl of 5 M NaCl and 150 µl of Ethanol (precooled to -20°C) the DNA is precipitated by incubation for 15 min. on ice and pelleted by centrifugation for 10 min. at 13,000 rpm and 4°C. The pellet is washed with 100 µl of 70% Ethanol (precooled to -20°C) and 0.2 M NaCl, centrifuged again and dried under vacuum. The pellets are dissolved in 50 µl Tris/EDTA (10 mM/0.1 mM; pH 7.5). 5 µl of the sample are spotted into a well of a nylon membrane bottomed white microwell plate (Pall Filtrationstechnik GmbH, Dreieich, FRG, product no: SM045BWP). The DNA is fixed to the membrane by baking for 10 min. at 70°C. The DNA loaded wells are filled with 100 µl of 0.45 µm filtrated 1 % blocking solution (maleic acid, 100 mM; NaCl, 150 mM; casein, 1 % (w/v); pH 7.5). All following incubation steps are done at room temperature. After incubation for 2 min. the solution is sucked through the membrane with a suitable vacuum manifold at -0.4 bar. After repeating the washing step once the wells are filled with 100 µl of a 1:10,000-dilution of Anti-digoxigenin-AP Fab fragments

(Boehringer Mannheim, FRG, No: 1 093 274) diluted in the blocking solution described above. After incubation for 2 min. and sucking the solution through the membrane, this step is repeated once. The wells are washed twice under vacuum with 200 µl washing-buffer 1 (maleic-acid, 100 mM; NaCl, 150 mM; Tween™ 20, 0.3 % (v/v); pH 7.5) After washing for
 5 another two times under vacuum with 200 µl washing-buffer 2 (Tris-HCl, 10 mM; NaCl, 100 mM; MgCl₂, 50 mM; pH 9.5) the wells are incubated for 5 min. with 50 µl of CSPD™ (Boehringer Mannheim, No: 1 655 884), diluted 1:100 in washing buffer 2 which serves as a chemiluminescent substrate for the subsequent alkaline phosphatase reaction.

10 The solution is sucked through the membrane and after 10 min. incubation the RLU/s (Relative Light Unit per second) are detected in a Luminometer e.g. MicroLumat LB 96 P (EG&G Berthold, Wilbad, FRG).

In order to correlate the relative light units to the polymerase units as defined commonly, a
 15 standard curve was prepared using a serial dilution of Taq DNA polymerase as a standard enzyme. The Taq polymerase was assayed in the buffer recommended by the supplier. The linear range of the standard curve was used to determine the relative activity of the *T. gorgonarius* DNA polymerase preparations.

20 The active fractions were pooled, dialyzed twice against 500 ml Buffer B and applied to a Fractogel TSK AF-Blue column (1x10; 7.8 ml bed volume) equilibrated with buffer B. After washing with 15 ml buffer B the column was eluted with a linear gradient of 156 ml from 0 to 3 M NaCl in buffer B supplemented with 0.05 % Thesit. The active fractions were pooled and dialyzed against the storage buffer C (20 mM Tris-HCl, pH 8.2; 10 mM 2-mercaptoethanol;
 25 0.1 mM EDTA; 50 mM (NH₄)₂SO₄; 50 % glycerol). After adding of 0.5 % of Nonidet™ P 40 (v/v) and 0.5 % of Thesit™ (v/v) the preparation was stored at -20°C.

Characterisation of the recombinant DNA Polymerase from *Thermococcus gorgonarius*

30 Recombinant and native *T. gorgonarius* DNA polymerase had the same apparent molecular weight when electrophoresed in 8 - 25 % SDS-PAGE gradient gels. Recombinant *T. gor-*

gonarius polymerase maintains the heat stability of the native enzyme. Recombinant *T. gorgonarius* polymerase has the same 3'-5' exonuclease activity as native *T. gorgonarius* polymerase, which is also sensitive to inhibition by an excess of dNTPs.

5

Example 5

Thermostability of *T. gorgonarius* DNA Polymerase

- 10 The thermostability of the DNA polymerase from *T. gorgonarius* purified as described in Example I was determined as follows: 5 units purified *T. gorgonarius* polymerase were incubated at 95°C in 100 µl of the following buffer: 50 mM Tris-HCl, pH 8.8 (at 25°C); 15 mM (NH₄)₂SO₄; 7 mM MgCl₂; 10 mM 2-mercaptoethanol; 200 µM each of dATP, dGTP, dCTP and dTTP; 0.1 % Nonidet P40, 0.1 % Thesit; 25 µg DNase treated calf thymus DNA.
- 15 15 µl samples were taken at 0, 5, 10, 15, 30, 45, 60 and 120 minutes. The remaining polymerase activity was measured as described in example IV by determining incorporation of labeled ³H-TTP into DNA in a 50 µl volume of the incubation mixture described above containing in addition 150 nCi of ³H-TTP. After incubation at 72°C for 30 minutes the reactions were stopped by addition of 300 µl 10 % TCA, and after 10 minutes at 0°C the mixtures were
- 20 applied onto 3MM filters (Whatman). The filters were washed three times with approximately 10 ml 5 % TCA each time, dried for 10 minutes at 75°C and the DNA bound radioactivity of each filter was measured in 5 ml scintillation liquid in a scintillation vial in LKB rack beta 1217/1218 (Pharmacia).
- 25 As shown in figure No. 4 the *T. gorgonarius* DNA polymerase retained almost 90 % of its initial activity after incubation for 120 minutes at 95°C, Pwo polymerase has a similar stability, while Taq DNA polymerase has a remaining activity of approximately 16 % only.

Example 6

Determination of 3'-5' proofreading activity

5 A series of units of *T. gorgonarius* DNA polymerase (see figure 5) were incubated for 4 hours at 72°C with 1 µg DNA molecular weight marker VI (Boehringer Mannheim) in the presence and absence of dNTP's, 1 mM each, in 50 µl of the following incubation buffer: 50 mM Tris-HCl, pH 7.8; 10 mM MgCl₂; 7 mM 2-mercaptoethanol with Paraffin overlay. After addition of 10 µl stop solution the DNA fragments were separated on a 1 % agarose gel. In the
10 absence of dNTP's a smear of DNA fragments or no DNA could be detected while in presence of dNTP's the DNA fragments remained undegraded.

Example 7

15

Fidelity of *T. gorgonarius* DNA polymerase in the PCR process

The fidelity of *T. gorgonarius* DNA polymerase in the PCR process was determined in an assay based on the amplification, circularisation and transformation of the pUC19 derivate
20 pUCIQ17, containing a functional *lac I*^q allele (Frey, B. and Suppmann B. (1995) *Biochemica* 2:34-35). PCR-derived mutations in *lac I* are resulting in a derepression of the expression of *lac Zα* and subsequent formation of a functional β-galactosidase enzyme which can be easily detected on X-Gal indicator plates. The error rates determined with this *lac I*-based PCR fidelity assay were in the range of 3.4 to 2.2 · 10⁻⁶.

25

The plasmid pUCIQ17 was linearized by digestion with DraII to serve as a substrate for PCR amplification with DNA polymerase of *T. gorgonarius*. Both of the primers used have ClaI sites at their 5 prime ends:

30

Primer 1: 5'-AGCTTATCGATGGCACTTTTCGGGGAAATGTGCG-3'

Primer 2: 5'-AGCTTATCGATAAGCGGATGCCGGGAGCAGACAAGC-3'

The length of the resulting PCR product is 3493 pb.

5 The PCR was performed in a final volume of 50 μ l in the presence of 1.5 mM $MgCl_2$, 50 mM Tris-HCl, pH 8.5 (25°C), 12.5 mM $(NH_4)_2SO_4$, 35 mM KCl, 200 μ M dNTPs and 2.5 units of *T. gorgonarius* DNA polymerase. Conditions of the amplification reaction using *T. gorgonarius* DNA polymerase are

10 The cycle conditions were as follows:

1 x denaturation of template for 2 min. at 95°C

8 x $\left[\begin{array}{l} \text{denaturation at 95°C for 10 sec.} \\ \text{annealing at 57°C for 30 sec.} \\ \text{elongation at 72°C for 4 min.} \end{array} \right.$

16 x $\left[\begin{array}{l} \text{denaturation at 95°C for 10 sec.} \\ \text{annealing at 57°C for 30 sec.} \\ \text{elongation at 72°C for 4 min.} \\ \text{+ cycle elongation of 20 sec. for each cycle} \end{array} \right.$

15

After PCR, the PCR products were PEG-precipitated (Barnes, W. M. (1992) *Gene* 112:229) the DNA restricted with ClaI and purified by agarose gel electrophoresis. The isolated DNA was ligated using the Rapid DNA Ligation Kit (Boehringer Mannheim GmbH) and the ligation products transformed in *E. coli* DH5 α , plated on TN Amp X-Gal plates. The α -comple-
 20 menting *E. coli* strain DH5 α transformed with the resulting plasmid pUCIQ17 (3632 bp), shows white ($lacI^+$) colonies on TN plates (1.5 % Bacto Tryptone, 1 % NaCl, 1.5 % Agar) containing ampicillin (100 μ g/ml) and X-Gal (0.004 % w/v). Mutations result in blue colonies.

After incubation overnight at 37°C, blue and white colonies were counted. The error rate (f) per bp was calculated with a rearranged equation as published by Keohavong and Thilly (Keohavong, P. and Thilly, W. (1989) *PNAS USA* 86:9253):

$$f = -\ln F / d \times b \text{ bp}$$

where F is the fraction of white colonies:

$$F = \text{white (lacI+)} \text{ colonies} / \text{total colony number};$$

d is the number of DNA duplications:

$$2^d = \text{output DNA} / \text{input DNA};$$

and b is the effective target size of the (1080bp) *lac I* gene, which is 349 bp according to Provost et al. (Provost et al. (1993) *Mut. Res.* 288:133).

Example 8

Fidelity assay

Determination of the misincorporation rates of DNA polymerases from *Pyrococcus furiosus* and *Thermococcus gorgonarius* under PCR conditions.

Error rates of many DNA polymerases are published. For example for the DNA polymerase of *Pyrococcus furiosus* various error rates were measured (Lit. 1-5). They may vary with the conditions used e.g. nucleotide triphosphate concentrations, enzyme preparation, buffer conditions and of course with the method used, the determination of the number of duplications and the way to calculate the misincorporation rate.

Therefore, the DNA polymerases *Pfu* (Stratagene) and *Tgo* (Boehringer Mannheim GmbH) were analyzed in parallel in the same system (Protocol: Frey, B. and Suppman, B. Boehringer Mannheim Biochemica Information, Nr. 96-1995, 21-23).

5 Table 1:

Fidelity of *Pfu* and *Tgo* DNA polymerases in PCR fidelity assay

DNA Polymerase	Plaques scored		Mutation	Error rate (a)	Error rate (b)
	Total	Mutant	frequency		
<i>Pfu</i>					
1. sample	3082	76	2.47	1,56 x 10 ⁻⁵	8,2 x 10 ⁻⁶
2. sample	2693	68	2.52	1,6 x 10 ⁻⁵	8,4 x 10 ⁻⁶
<i>Tgo</i>					
1. sample	1904	12	0.63	3,5 x 10 ⁻⁶	1,8 x 10 ⁻⁶
2. sample	2003	20	1	5,6 x 10 ⁻⁶	2,9 x 10 ⁻⁶

- 10 (a) Error rate calculated according to the equation used by Stratagene (Lundberg K.S. et al. (1991) Gene 108, 1-6).

$$ER = mf / bp \times d$$

- 15 ER = error rate

mf = mutation frequency in % minus background frequency of 0.0017 %

bp is the number of detectable sites in the *lac I* gene sequence (182)

d is the number of duplications. In this particular experiment the number of duplications was determined/estimate for *Pfu* to be 8,64 and for *Tgo* to be 9,64

20

- (b) Error rate calculated per bp with a rearranged equation published by Keohavong P. and Thilly W. (1989) *PNAS USA* **86**, 9253.

$$ER = -\ln F / d \times b \text{ bp}$$

F = fraction of white colonies (white colonies / total number of colonies)

d = the number of duplications. 2^d = output DNA / input DNA

b is the effective target size of the (1080 bp) *lac I* gene, which is 349 bp according to Provost, G.S., Kretz, P.L., Hammer, R.T., Matthews, C.D., Rogers, B.J., Lundberg K., S., Dycaico, M.J. and Short, J.M. (1993) *Mut. Res.* **288**, 133 ff.

10 Result:

These data show that the mutation frequency of *Tgo* DNA polymerase is lower than that of *Pfu*, and the fidelity (calculated in errors per base pair) is higher no matter which way of calculation was used.

15

References describing error rates for *Pfu*:

1. Lundberg, K.S., Shoemaker, D.D., Adams M.W.W., Short, J.M., Sorge, J.A. and 2 Mathur, E.J. (1991) *Gene* **108**, 1-6. (1.6×10^{-6} errors/base)

20

2. Flaman, J.-M., Frebourg, T., Moreau, V., Charbonnier, F., Martin, C., Ishioka, C., Friend, S.H. and Iggo, R. (1994) *NAR* **22**, 3259-3260. (2×10^{-6} errors/base) For Tli (Vent) Polymerase: (Variations in error rate depending on assay)

25 3. Cariello, N.F., Swenberg, J.A. and Skopek, T.R. (1991) *NAR* **19**, 4193-4198. (2.4×10^{-5} errors/base)

4. Ling, L.L., Keohavong, P., Dias, C. and Thilly, W.G. (1991) *PCR Methods Appl.* **1**, 63-69. (4.5×10^{-5} errors/base)

30

5. Matilla, P., Korpela, J., Tenkanen, T. and Pitkanen, K. (1991) *NAR* **19**, 4967-4973. (5.7×10^{-5} errors/base)

Claims

1. A purified thermostable DNA polymerase obtainable from *Thermococcus gorgonarius*.
- 5 2. A purified thermostable DNA polymerase according to claim 1 which catalyses the template directed polymerisation of DNA and retains about 90 % of its activity after incubation for two hours at about 95°C in the presence of a stabilizer.
3. Enzyme according to claims 1 or 2, wherein said polymerase is characterized by a at least a two-fold greater replication fidelity than DNA polymerase obtainable from *Pyrococcus*
- 10 *furiosus*.
4. The polymerase as claimed in any one of claims 1 - 3, wherein said polymerase has an apparent molecular weight between about 92 000 to 96 000 daltons.
5. The polymerase as claimed in any one of claims 1 - 4, wherein said polymerase is obtainable from E.coli.
- 15 6. A stabilized composition consisting of a polymerase as claimed in any one of claims 1 - 5 and a stabilizer.
7. The composition according to claims 1 - 6, wherein said stabilizer is a non-ionic detergent.
8. The polymerase as claimed in any one of claims 1 - 7, wherein said polymerase is characterized by having 3'-5'-exonuclease (proofreading) activity.
- 20 9. The composition according to claim 1 - 8, wherein Thesit and/or Nonident P 40 serve as stabilizer.
10. An isolated DNA sequence coding for the polymerase according to any one of claims 1 - 9 obtainable from *Thermococcus gorgonarius*.
- 25 11. An isolated DNA sequence coding for the polymerase as claimed in claim 10 contained within the plasmid pBTac2Tgo.
12. An isolated DNA sequence of claim 11 contained within an approximately 2.3 kB EcoRI/PstI restriction fragment of plasmid pBTac2Tgo.
13. An isolated DNA sequence represented by the formula shown in SEQ ID No. 6.

14. A vector containing the isolated DNA sequence as claimed in any one of claims 10 - 13.
15. The vector of claim 14, wherein such vector is plasmid pBTac2Tgo.
16. The vector according to claims 14 and 15 providing some or all of the following features:
 - (1) promoters or sites of initiation of transcription
 - 5 (2) operators which could be used to turn gene expression on or off
 - (3) ribosome binding sites for improved translation
 - (4) transcription or translation termination sites.
17. A microbial host transformed with the vector of claims 14 - 16.
18. A microbial host according to claim 17 wherein said host is from *E. coli* LE 392 pUBS
10 520 and designated *E. coli* pBtac2Tgo.
19. A process for the preparation of DNA polymerase according to any one of claims 1 - 9 comprising the steps:
 - (a) culturing the natural strain *Thermococcus gorgonarius*
 - (b) suspending the cells of the natural cells in buffer
 - 15 (c) disrupting the cells
 - (d) purifying the DNA polymerase by several chromatographic steps.
20. A process for the preparation of DNA polymerase according to any one of claims 1 - 9 comprising growing a microbial host strain according to claims 17 or 18 and purifying the DNA polymerase therefrom.
- 20 21. A process for amplifying DNA, characterized in that a thermostable DNA polymerase according to any one of claims 1 - 9 is used.
22. A process for DNA sequencing or DNA labelling, characterized in that a thermostable DNA polymerase wherein the 3'-5' exonuclease activity of said DNA polymerase is inactivated.
- 25 23. A process for second cDNA cloning and DNA sequencing, characterized in that a thermostable DNA polymerase according to any one of claims 1-9 is used.
24. A process for DNA sequencing, characterized in that a thermostable DNA polymerase according to any one of claims 1-9 is used.

Abstract

A purified thermostable enzyme is derived from the thermophilic archaeobacterium *Thermococcus gorgonarius*. The enzyme can be native or recombinant, retains approximately 90 % of its activity after incubation for two hours at 95°C in the presence of stabilizing agents and possesses 3'-5' proofreading exonuclease activity. Thermostable DNA polymerases are useful in many recombinant DNA techniques, especially nucleic acid amplification by the polymerase chain reaction (PCR).

Figure 1:

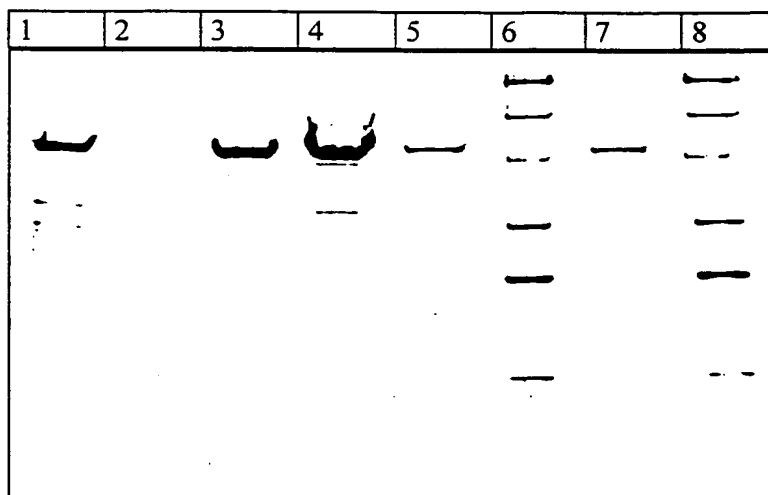


Figure 2:

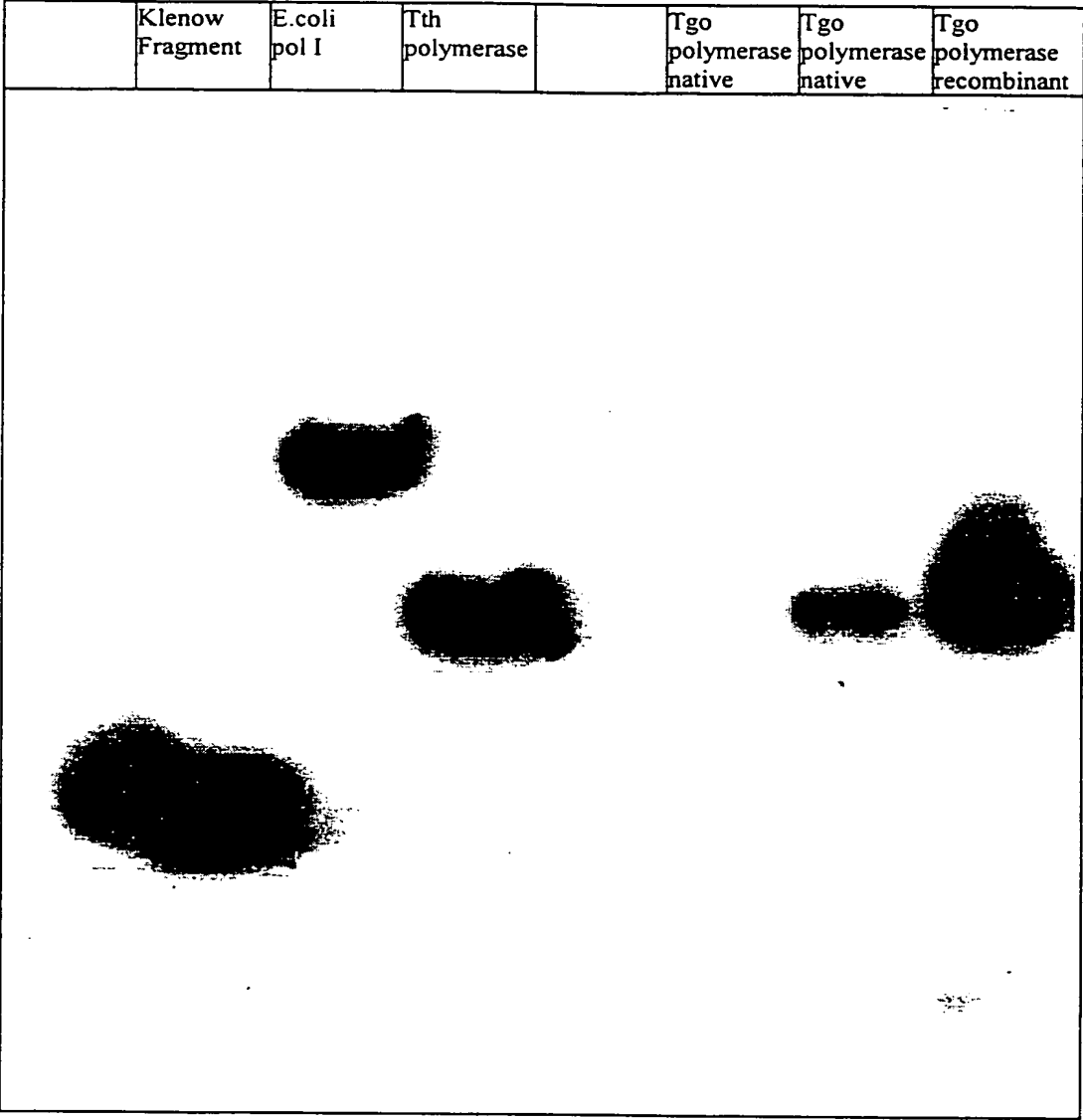


Figure 3:

SEQ ID NO. 6:

ATG ATC CTC GAT ACA GAC TAC ATA ACT GAG GAT GGA AAG CCC GTC ATC 48
TAC TAG GAG CTA TGT CTG ATG TAT TGA CTC CTA CCT TTC GGG CAG TAG 48
M I L D T D Y I T E D G K P V I 16
AGG ATC TTC AAG AAG GAG AAC GGC GAG TTC ACC ATA GAC TAC GAC AGA 96
TCC TAG AAG TTC TTC CTC TTG CCG CTC AAG TGG TAT CTG ATG CTG TCT 96
R I F K K E N G E F T I D Y D R 32
AAC TTT GAG CCA TAC ATC TAC GCG CTC TTG AAG GAC GAC TCT CCG ATT 144
TTG AAA CTC GGT ATG TAG ATG CGC GAG AAC TTC CTG CTG AGA GGC TAA 144
N F E P Y I Y A L L K D D S P I 48
GAG GAC GTC AAG AAG ATA ACT GCC GAG AGG CAC GGC ACT ACC GTT AGG 192
CTC CTG CAG TTC TTC TAT TGA CGG CTC TCC GTG CCG TGA TGG CAA TCC 192
E D V K K I T A E R H G T T V R 64
GTT GTC AGG GCC GAG AAA GTG AAG AAG AAG TTC CTA GGC AGG CCG ATA 240
CAA CAG TCC CGG CTC TTT CAC TTC TTC TTC AAG GAT CCG TCC GGC TAT 240
V V R A E K V K K K F L G R P I 80
GAG GTC TGG AAG CTC TAC TTC ACT CAC CCC CAG GAC GTT CCC GCA ATC 288
CTC CAG ACC TTC GAG ATG AAG TGA GTG GGG GTC CTG CAA GGG CGT TAG 288
E V W K L Y F T H P Q D V P A I 96

AGG GAC AAG ATA AAG GAG CAT CCT GCC GTT GTG GAC ATC TAC GAG TAC 336
 TCC CTG TTC TAT TTC CTC GTA GGA CGG CAA CAC CTG TAG ATG CTC ATG 336
 R D K I K E H P A V V D I Y E Y 112

 GAC ATC CCC TTC GCG AAG CGC TAC CTC ATA GAC AAA GGC TTA ATC CCG 384
 CTG TAG GGG AAG CGC TTC GCG ATG GAG TAT CTG TTT CCG AAT TAG GGC 384
 D I P F A K R Y L I D K G L I P 128

 ATG GAG GGC GAC GAG GAA CTT AAG ATG CTC GCC TTC GAC ATC GAG ACG 432
 TAC CTC CCG CTG CTC CTT GAA TTC TAC GAG CGG AAG CTG TAG CTC TGC 432
 M E G D E E L K M L A F D I E T 144

 CTC TAT CAC GAG GGC GAG GAG TTC GCC GAA GGG CCT ATC CTG ATG ATA 480
 GAG ATA GTG CTC CCG CTC CTC AAG CGG CTT CCC GGA TAG GAC TAC TAT 480
 L Y H E G E E F A E G P I L M I 160

 AGC TAC GCC GAC GAG GAA GGG GCG CGC GTT ATT ACC TGG AAG AAT ATC 528
 TCG ATG CGG CTG CTC CTT CCC CGC GCG CAA TAA TGG ACC TTC TTA TAG 528
 S Y A D E E G A R V I T W K N I 176

 GAC CTT CCC TAT GTC GAC GTC GTT TCC ACC GAG AAG GAG ATG ATA AAG 576
 CTG GAA GGG ATA CAG CTG CAG CAA AGG TGG CTC TTC CTC TAC TAT TTC 576
 D L P Y V D V V S T E K E M I K 192

 CGC TTC CTC AAG GTC GTC AAG GAA AAG GAT CCC GAC GTC CTC ATA ATC 624
 GCG AAG GAG TTC CAG CAG TTC CTT TTC CTA GGG CTG CAG GAG TAT TAG 624
 R F L K V V K E K D P D V L I I 208

GAA	CTC	GGA	AAA	GAG	TTC	TTC	CCT	ATG	GAA	GCC	CAG	CTC	TCG	CGC	CTC	1008
CTT	GAG	CCT	TTT	CTC	AAG	AAG	GGA	TAC	CTT	CGG	GTC	GAG	AGC	GCG	GAG	1008
E	L	G	K	E	F	F	P	M	E	A	Q	L	S	R	L	336
GTA	GGC	CAG	AGC	CTC	TGG	GAT	GTA	TCT	CGC	TCG	AGT	ACC	GGA	AAC	CTC	1056
CAT	CCG	GTC	TCG	GAG	ACC	CTA	CAT	AGA	GCG	AGC	TCA	TGG	CCT	TTG	GAG	1056
V	G	Q	S	L	W	D	V	S	R	S	S	T	G	N	L	352
GTC	GAG	TGG	TTT	TTG	CTG	AGG	AAG	GCC	TAC	GAG	AGG	AAT	GAA	CTT	GCA	1104
CAG	CTC	ACC	AAA	AAC	GAC	TCC	TTC	CGG	ATG	CTC	TCC	TTA	CTT	GAA	CGT	1104
V	E	W	F	L	L	R	K	A	Y	E	R	N	E	L	A	368
CCA	AAC	AAG	CCG	GAC	GAG	AGG	GAG	CTG	GCA	AGA	AGA	AGG	GAG	AGC	TAC	1152
GGT	TTG	TTC	GGC	CTG	CTC	TCC	CTC	GAC	CGT	TCT	TCT	TCC	CTC	TCG	ATG	1152
P	N	K	P	D	E	R	E	L	A	R	R	R	E	S	Y	384
GCG	GGT	GGA	TAC	GTC	AAG	GAG	CCC	GAA	AGG	GGA	CTG	TGG	GAG	AAC	ATC	1200
CGC	CCA	CCT	ATG	CAG	TTC	CTC	GGG	CTT	TCC	CCT	GAC	ACC	CTC	TTG	TAG	1200
A	G	G	Y	V	K	E	P	E	R	G	L	W	E	N	I	400
GTG	TAT	CTG	GAC	TTC	CGC	TCC	CTG	TAT	CCT	TCG	ATA	ATA	ATC	ACC	CAT	1248
CAC	ATA	GAC	CTG	AAG	GCG	AGG	GAC	ATA	GGA	AGC	TAT	TAT	TAG	TGG	GTA	1248
V	Y	L	D	F	R	S	L	Y	P	S	I	I	I	T	H	416
AAC	GTC	TCC	CCT	GAT	ACA	CTC	AAC	AGG	GAG	GGT	TGT	GAG	GAG	TAC	GAC	1296
TTG	CAG	AGG	GGA	CTA	TGT	GAG	TTG	TCC	CTC	CCA	ACA	CTC	CTC	ATG	CTG	1296
N	V	S	P	D	T	L	N	R	E	G	C	E	E	Y	D	432

TAC AAC GGC GAC AAC TTC GAC TTC GCC TAC CTC AAG AAG CGC TCC GAG	672
ATG TTG CCG CTG TTG AAG CTG AAG CGG ATG GAG TTC TTC GCG AGG CTC	672
Y N G D N F D F A Y L K K R S E	224
AAG CTC GGA GTC AAG TTC ATC CTC GGA AGG GAA GGG AGC GAA CCG AAA	720
TTC GAG CCT CAG TTC AAG TAG GAG CCT TCC CTT CCC TCG CTT GGC TTT	720
K L G V K F I L G R E G S E P K	240
ATC CAG CGC ATG GGC GAT CGC TTT GCG GTG GAG GTC AAG GGA AGG ATT	768
TAG GTC GCG TAC CCG CTA GCG AAA CGC CAC CTC CAG TTC CCT TCC TAA	768
I Q R M G D R F A V E V K G R I	256
CAC TTC GAC CTC TAC CCC GTC ATT AGG AGA ACG ATT AAC CTC CCC ACT	816
GTG AAG CTG GAG ATG GGG CAG TAA TCC TCT TGC TAA TTG GAG GGG TGA	816
H F D L Y P V I R R T I N L P T	272
TAC ACC CTT GAG GCA GTA TAT GAA GCC ATC TTT GGA CAG CCG AAG GAG	864
ATG TGG GAA CTC CGT CAT ATA CTT CGG TAG AAA CCT GTC GGC TTC CTC	864
Y T L E A V Y E A I F G Q P K E	288
AAG GTC TAC GCT GAG GAG ATA GCG CAG GCC TGG GAA ACG GGC GAG GGA	912
TTC CAG ATG CGA CTC CTC TAT CGC GTC CGG ACC CTT TGC CCG CTC CCT	912
K V Y A E E I A Q A W E T G E G	304
TTA GAA AGG GTG GCC CGC TAC TCG ATG GAG GAC GCG AAG GTA ACC TAT	960
AAT CTT TCC CAC CGG GCG ATG AGC TAC CTC CTG CGC TTC CAT TGG ATA	960
L E R V A R Y S M E D A K V T Y	320

GTG	GCT	CCT	CAG	GTA	GGC	CAT	AAG	TTC	TGC	AAG	GAC	TTC	CCC	GGC	TTC	1344
CAC	CGA	GGA	GTC	CAT	CCG	GTA	TTC	AAG	ACG	TTC	CTG	AAG	GGG	CCG	AAG	1344
V	A	P	Q	V	G	H	K	F	C	K	D	F	P	G	F	448
ATC	CCA	AGC	CTC	CTC	GGA	GAC	CTC	TTG	GAG	GAG	AGA	CAG	AAG	GTA	AAG	1392
TAG	GGT	TCG	GAG	GAG	CCT	CTG	GAG	AAC	CTC	CTC	TCT	GTC	TTC	CAT	TTC	1392
I	P	S	L	L	G	D	L	L	E	E	R	Q	K	V	K	464
AAG	AAG	ATG	AAG	GCC	ACT	ATA	GAC	CCA	ATC	GAG	AAG	AAA	CTC	CTC	GAT	1440
TTC	TTC	TAC	TTC	CGG	TGA	TAT	CTG	GGT	TAG	CTC	TTC	TTT	GAG	GAG	CTA	1440
K	K	M	K	A	T	I	D	P	I	E	K	K	L	L	D	480
TAC	AGG	CAA	CGA	GCA	ATC	AAA	ATC	CTT	GCT	AAT	AGC	TTC	TAC	GGT	TAC	1488
ATG	TCC	GTT	GCT	CGT	TAG	TTT	TAG	GAA	CGA	TTA	TCG	AAG	ATG	CCA	ATG	1488
Y	R	Q	R	A	I	K	I	L	A	N	S	F	Y	G	Y	496
TAC	GGC	TAT	ACA	AAG	GCC	CGC	TGG	TAC	TAC	AAG	GAG	TGC	GCC	GAG	AGC	1536
ATG	CCG	ATA	TGT	TTC	CGG	GCG	ACC	ATG	ATG	TTC	CTC	ACG	CGG	CTC	TCG	1536
Y	G	Y	T	K	A	R	W	Y	Y	K	E	C	A	E	S	512
GTT	ACC	GGT	TGG	GGC	AGG	GAG	TAC	ATC	GAG	ACC	ACG	ATA	AGG	GAA	ATA	1584
CAA	TGG	CCA	ACC	CCG	TCC	CTC	ATG	TAG	CTC	TGG	TGC	TAT	TCC	CTT	TAT	1584
V	T	G	W	G	R	E	Y	I	E	T	T	I	R	E	I	528
GAG	GAG	AAA	TTT	GGC	TTT	AAA	GTC	CTC	TAC	GCG	GAC	ACA	GAT	GGA	TTT	1632
CTC	CTC	TTT	AAA	CCG	AAA	TTT	CAG	GAG	ATG	CGC	CTG	TGT	CTA	CCT	AAA	1632
E	E	K	F	G	F	K	V	L	Y	A	D	T	D	G	F	544
TTC	GCA	ACA	ATA	CCT	GGA	GCG	GAC	GCC	GAA	ACC	GTC	AAA	AAG	AAG	GCA	1680
AAG	CGT	TGT	TAT	GGA	CCT	CGC	CTG	CGG	CTT	TGG	CAG	TTT	TTC	TTC	CGT	1680
F	A	T	I	P	G	A	D	A	E	T	V	K	K	K	A	560

AAG GAG TTC CTG GAC TAC ATC AAC GCC AAA CTG CCC GGC CTG CTC GAA 1728
TTC CTC AAG GAC CTG ATG TAG TTG CGG TTT GAC GGG CCG GAC GAG CTT 1728
K E F L D Y I N A K L P G L L E 576

CTC GAA TAC GAG GGC TTC TAC AAG CGC GGC TTC TTC GTG ACG AAG AAG 1776
GAG CTT ATG CTC CCG AAG ATG TTC GCG CCG AAG AAG CAC TGC TTC TTC 1776
L E Y E G F Y K R G F F V T K K 592

AAG TAC GCG GTT ATA GAC GAG GAG GAC AAG ATA ACG ACG CGC GGG CTT 1824
TTC ATG CGC CAA TAT CTG CTC CTC CTG TTC TAT TGC TGC GCG CCC GAA 1824
K Y A V I D E E D K I T T R G L 608

GAA ATA GTT AGG CGT GAC TGG AGC GAG ATA GCG AAG GAG ACG CAG GCG 1872
CTT TAT CAA TCC GCA CTG ACC TCG CTC TAT CGC TTC CTC TGC GTC CGC 1872
E I V R R D W S E I A K E T Q A 624

AGG GTT CTT GAG GCG ATA CTA AAG CAC GGT GAC GTT GAA GAA GCG GTA 1920
TCC CAA GAA CTC CGC TAT GAT TTC GTG CCA CTG CAA CTT CTT CGC CAT 1920
R V L E A I L K H G D V E E A V 640

AGG ATT GTC AAA GAG GTT ACG GAG AAG CTG AGC AAG TAC GAG GTT CCA 1968
TCC TAA CAG TTT CTC CAA TGC CTC TTC GAC TCG TTC ATG CTC CAA GGT 1968
R I V K E V T E K L S K Y E V P 656

CCG GAG AAG CTG GTC ATC TAC GAG CAG ATA ACC CGC GAC CTG AAG GAC 2016
GGC CTC TTC GAC CAG TAG ATG CTC GTC TAT TGG GCG CTG GAC TTC CTG 2016
P E K L V I Y E Q I T R D L K D 672

TAC AAG GCC ACC GGG CCG CAT GTG GCT GTT GCA AAA CGC CTC GCC GCA	2064
ATG TTC CGG TGG CCC GGC GTA CAC CGA CAA CGT TTT GCG GAG CGG CGT	2064
Y K A T G P H V A V A K R L A A	688
AGG GGG ATA AAA ATC CGG CCC GGA ACG GTC ATA AGC TAC ATC GTG CTC	2112
TCC CCC TAT TTT TAG GCC GGG CCT TGC CAG TAT TCG ATG TAG CAC GAG	2112
R G I K I R P G T V I S Y I V L	704
AAA GGC TCG GGA AGG ATT GGG GAC AGG GCT ATA CCC TTT GAC GAA TTT	2160
TTT CCG AGC CCT TCC TAA CCC CTG TCC CGA TAT GGG AAA CTG CTT AAA	2160
K G S G R I G D R A I P F D E F	720
GAC CCG GCA AAG CAC AAG TAC GAT GCA GAA TAC TAC ATC GAG AAC CAG	2208
CTG GGC CGT TTC GTG TTC ATG CTA CGT CTT ATG ATG TAG CTC TTG GTC	2208
D P A K H K Y D A E Y Y I E N Q	736
GTT CTT CCA GCT GTG GAG AGG ATT CTG AGG GCC TTT GGT TAC CGT AAA	2256
CAA GAA GGT CGA CAC CTC TCC TAA GAC TCC CGG AAA CCA ATG GCA TTT	2256
V L P A V E R I L R A F G Y R K	752
GAA GAT TTA AGG TAT CAG AAA ACG CGG CAG GTT GGC TTG GGG GCG TGG	2304
CTT CTA AAT TCC ATA GTC TTT TGC GCC GTC CAA CCG AAC CCC CGC ACC	2304
E D L R Y Q K T R Q V G L G A W	768
CTA AAA CCT AAG ACA TGA	2322
GAT TTT GGA TTC TGT ACT	2322
L K P K T *	773

Figure 4:

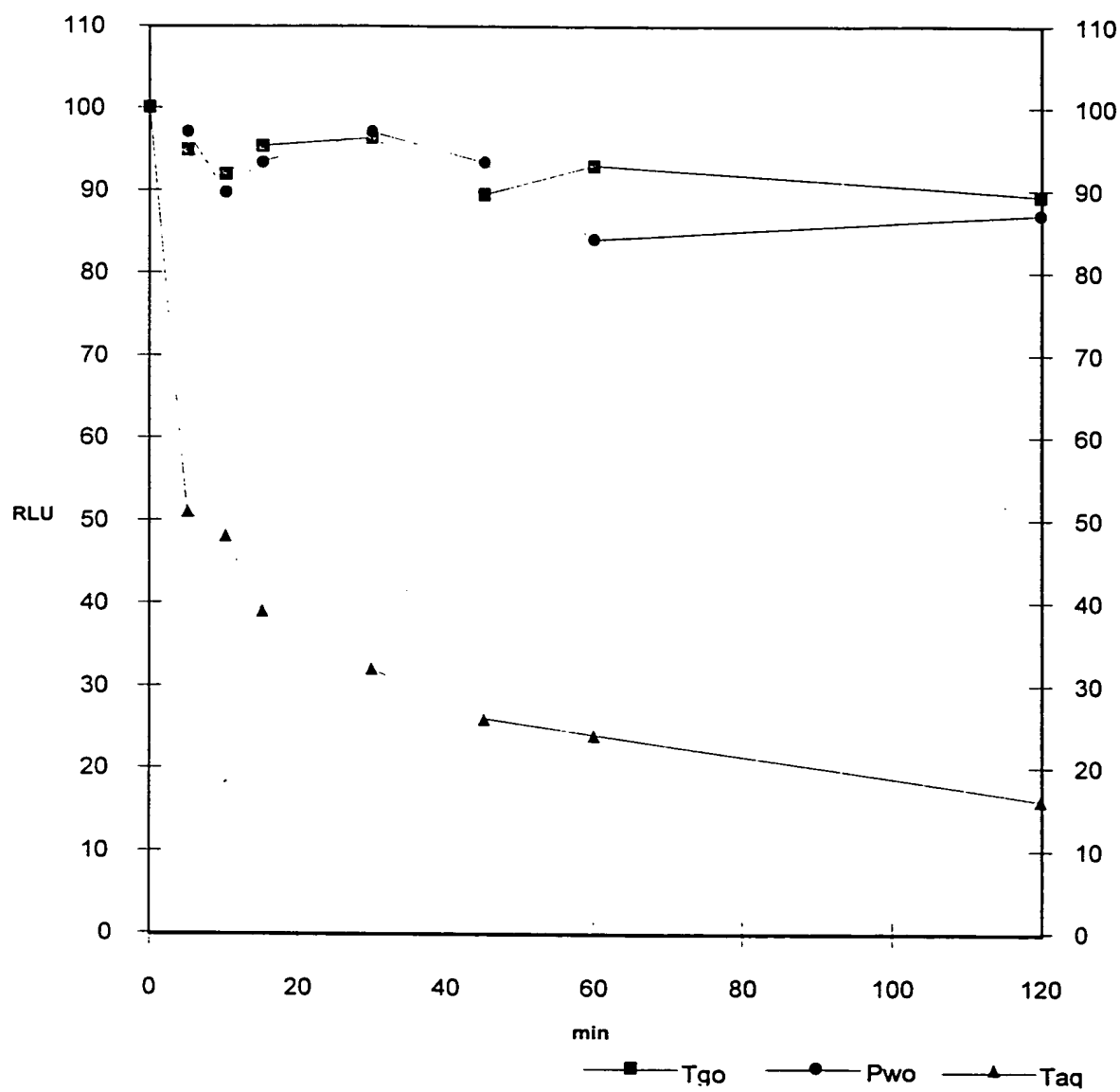
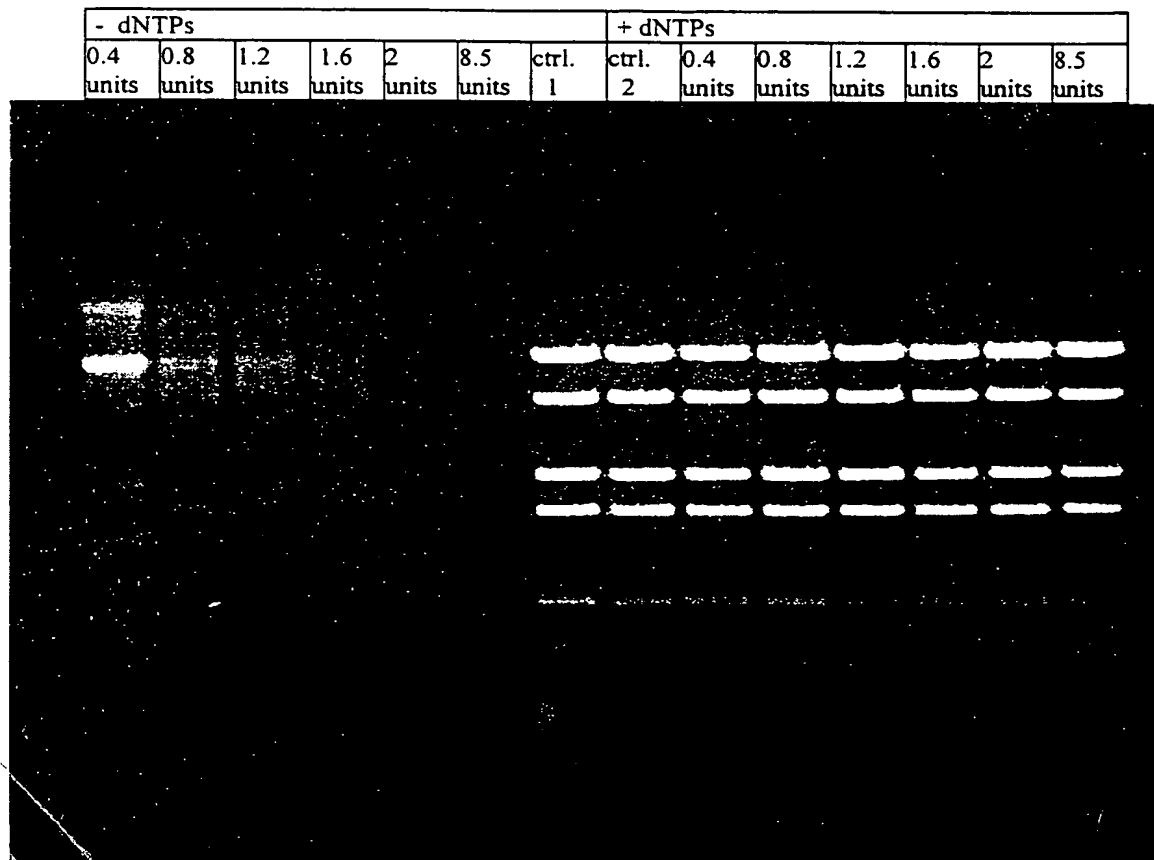


Figure 5:





100



**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ BLACK BORDERS
- ☒ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☒ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☒ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

This Page Blank (uspto)